ImageStream User Guide (N.C 03.044)

Booking

You have to book the ImageStream with your user name on the webpage:
https://ppms.eu/lmu/start/

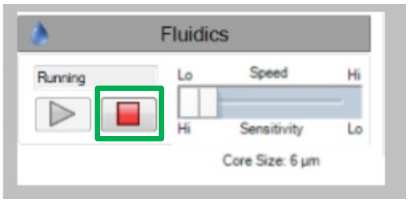
Problems

Any problems must be reported asap as 'Incident' on https://ppms.eu/lmu/start/

Start-up

1. **(i)** Press the green power button inside the front door of the instrument, **(ii)** turn on the large and **(iii)** small computer.
2. Log in to **Amnis** account.
3. Launch the **ISX** application. Do not close the opening windows.
4. Ensure SpeedBeads tube is loaded on the bead port, at least 1ml. When installing a fresh tube of SpeedBeads, the leftover beads can be added to this new tube.
5. Verify that the buffer containers are full, or fill up as needed:
   SpeedBeads
   Sheath = PBS Rinse = dH20
   cleanser = Coulter Clenz
   Sterilizer = 0,5% bleach (Hypochlorite),
   Debubbler = 70% Isopropanol

   - SpeedBeads in the fridge (make sure there is always one fresh vial at RT)
   - dH20 and PBS next to the fridge
   - CoulterClenz is at the sink
   - Sterilizer (bleach) and Debubbler (Isoprop) are in the steel safety cabinet.
6. Empty the **Waste** tank into the sink.
7. Select **Start Up** and the instrument will load sheath in ~14min; check "Calibrate with ASSIST", then it will be done automatically after Start Up is completed.
8. If ASSIST was not run in the previous step, click "**Start All Calibration and Test"** in the calibration window
   => Whole start up and calibration takes about 45min to complete.
9. When all tests pass, ASSIST light is green and system is ready to run. Close the Calibration window. If Assist light is red (one or more calibration tests failed), check the troubleshooting section below.
10. If there will be a break after ASSIST, stop the fluidics by clicking on the "STOP" button in the Fluidics tab.



Acquisition

1. In case fluidics are off, initiate fluidics by clicking on the "Play" button in the Fluidics tab.
2. Select file and load **default template**, for opening saved template, choose **load template**.
3. Before running any of your samples, load a tube containing 50ul of 10% bleach, run it for 5min and then 75ul dH_20 for 5 min.
4. Choose the objective: 20X / 40X / 60X under **Magnification**
5. For spot counting applications such as FISH, Autophagy, nuclear translocation, select the EDF (extended depth of field) option.

6. Under **Illumination**, turn on the appropriate lasers for the used fluorochromes.
7. *Facultative*: select only the needed channels for your fluorochromes by clicking on a channel column heading (i.e. Ch2) and checking the "collected" check box to save the required channel. (Unused channels can also be dropped later when loading the data for analysis.)
8. Default Channels for Brightfield images are Ch1&Ch9, but this can be changed if Ch1/Ch9 are needed for fluorophores. If needed, a specific SSC channel can be activated (~2-5mW in Ch6 or Ch12).
9. Under **Fluidics**, ensure that speed is set to low.
10. Press load and place the sample tube with all of the fluorochromes being used and the brightest fluorescence. **Attention: avoid air bubbles!**
11. Wait until fluidics stabilize, and images appear focused. The event rate should be 8001000 events per second, if not see troubleshooting. Under **Focus and Centering**, center the core stream images if necessary.
12. Check the saturation in all channels, **Raw Max Pixel intensity** should be visualized as histogram, all data points should be within 100-4000, a peak at 4000 is a sign of saturation, turn down laser power until a maximum of 1% of events are saturated.
13. Make a folder to export your data into => do not store any data on the PC! Always save/export to an external hard drive.
14. Select the acquisition parameters under **File Acquisition**: enter file name, choose your file destination, number of events, and region to collect
15. You may manually adjust image display settings:
    a. click on the **display setting in image gallery** tool  => select the channel by clicking on the channel name => adjust the right and left green bars in the graph, by clickdragging the vertical green line on the left side allows you to set the display pixel to 0, and click-dragging the vertical green line on the right side allows you to set the display pixel intensity to 255 => click "**OK**".
    b. for setting image display automatically, using wizard, click on the wand  select the channel to set and click finish.
16. Create gating strategy;
    a. Scatterplot of Area vs Aspect Ratio of Brightfield channel to gate on single cells (or doublets as needed) and eliminate SpeedBeads and debris.
    b. Gate on focused cells in a Gradient RMS Brightfield (or other useful channel depending on application) histogram – cells with highest Gradient RMS value are best in focus.
    c. Thereafter, create dot plots and regions to identify the cells to collect. Plot intensity of interested Channels to gate on a subpopulation of cells.

Collecting and saving the data

1. As needed save the raw image files **(.rif)** as well **as .fcs** files **–** this can be selected in the File menu.
2. Click "**'Acquire**" to begin saving events to a file.
3. When acquisition finished, click "**Return**", then remove the sample and click "**OK**" – a flush will follow.
4. Load next sample by clicking "**Load**".

5. After acquiring samples, run single color compensation controls, see Compensation section.
6. Save your settings as a template by selecting "**Save Template"** from the File menu.

Compensation

For creating a new compensation matrix, you can either use the compensation Wizard or acquire samples manually. Compensation matrix can be calculated in INSPRE, or afterwards in IDEAS software. To wash out DNA dyes or other sticky dyes, and avoiding carry-over between samples (which will falsify the compensation), load 50ul of 10% bleach and then 75ul of dH20 for 2-5min.

1. To **manually** collect compensation controls, turn off Brightfield and SSC laser, verify all channels are on, and collect 1000 events (suitable region of positive cells!) for each compensation control sample. Label each file manually and acquire.
   OR
2. For opening the Wizard**,** click in the analysis tools and choose "Compensation".
3. Press "Load" and insert the compensation control sample, press "Next", verify the identified channel is the correct one. (If it is not, it is a bad sign for the quality of the compensation control sample.)
4. If not all the cells are positive for the marker, draw a region on the intensity scatter plot to define the positive population.
5. As before, set the acquisition file name, destination/population to save, press "Acquire". 6. Click "Return" / "Load" to continue with the next single color control
7. Repeat steps 3-6 for each compensation sample.
8. For each sample reset the Image gallery population to view All, and then create an appropriate population for each sample.
9. Click "Exit" when done to save the compensation matrix.

Cleaning after yourself / between users

1. Load 200ul Bleach, let run for 5min, at "high" speed.
2. Load 200ul dH2O, let run for 5min at "high" speed.
3. If the next usage will be within 2 hours, load 200µl if dH20 and run the machine on minimum speed. Otherwise fluidics are stopped automatically after unloading the tube.

Last User of the day

4. Remove any tubes from the uptake port.
5. Click **"Shutdown"** - it takes about 45 minutes to sterilize the system, then automatically turns off everything.

ASSIST Test Troubleshooting guide:

Next to each calibration test button is a green or red rectangle. If the procedure fails, it turns red. **If a procedure fails, repeat it individually**: click on the test and then "start" in the window that opens. If it fails twice, see below for troubleshooting.
Please always report these cases to us as well, so that we can take action if needed!

If 60x camera synchronization calibration fails, please change manually to 60x magnification, wait until the speed beads are running stably and re-run the calibration individually.

Symptom	Possible Causes	Recommended Solutions
Instrument will not pass ASSIST	Incorrect template loaded	Go to the file drop down and select "load default template". Re-run ASSIST.
	SpeedBeads fail to run	Verify the beads will run by returning any sample, going to fluidics section and press stop, then run. Next go to the advanced drop down, select flow speed, and check that the red and black histograms have tight CVs at the appropriate core velocity. To view bead images select the All population and check include beads.
	SpeedBeads are not running properly	The particles must be running >1000 events per second, and without significant clumping. If the beads are diluted or clumped, try running a fresh tube of beads. If the problem persists there may be a fluidics issue, see the Flow rate stops or slows over time section.
	Side Scatter Calibration failure	Verify the 785 SSC laser turns on and can set power properly. Completely power down the instrument and power back up to re-run the test. Verify spatial offsets passed.
	Laser Power test failure	Verify the laser turns on and can set power properly. Completely power down the instrument and power back up to re-run the test. Verify spatial offsets and frame offsets passed.
	Brightfield alignment test failure	Verify brightfield is working properly.
	Brightfield uniformity test failure	Verify brightfield is working properly.
	Camera noise test failure	Verify camera can image properly. Completely power down the instrument and power back up to re-run the test.
	Flow Core Axial Stability test failure	Verify the reagent buffers are full. Run the sterilize script followed by the startup script, and re-run the test. *See solutions for Unstable fluidics* .

	Flow Core Lateral Stability test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Flow Core Position test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Focus Percentage test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Focus Uniformity test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Image Quality test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Flow Core Position test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Focus Percentage test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Focus Uniformity test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Image Quality test failure	See solution above (*see Flow Core Axial Stability test failure*).
	Dark Current calibration failure	Make sure the excitation lasers are off and brightfield is blocked. Completely power down the instrument and power back up to re-run the test.
	Brightfield XTalk calibration failure	Verify brightfield is working properly, and that spatial offsets passed.
	Horizontal Laser Calibration failure	Verify the laser turns on and can set power properly. Completely power down the instrument and power back up to re-run the test. Verify spatial offsets passed.
	Retro Calibration failure	Verify the laser turns on and can set power properly. Verify spatial offsets and frame offsets passed.

Further Troubleshooting guide:

Symptom	Possible Causes	Recommended Solutions
INSPIRE fails to launch	Splash screen is not responding	On the keyboard press Ctrl-Alt- Delete, open the task manager, select INSPIRE and press end task. Wait 60 seconds and try restarting INSPIRE.
	Loss of communication between the computers and instrument.	Shut down the computer, and power off the instrument. Verify all computers are off. Power on the instrument and the computer, wait 5 min and try launching INSPIRE.
One channel saturates while the others do not	Instrument sensitivity is not optimized	The best instrument setup maximizes the dynamic range of fluorescence signal, while at the same time avoiding image pixel saturation (which cannot be compensated). In general decreasing the laser powers until no pixels saturate.
	Probing protocol requires better stain balance	Reduce the concentration of the stain that produces the saturating signal so that all probes can be simultaneously imaged without excessive saturation.
	Excessive fluorescent dye is left in the sample buffer.	Some DNA dyes are required to run with the sample to stain properly, however if too much dye is in solution it can cause the core stream to fluoresce. It's important to balance the concentration of these dyes so that the cells can be imaged properly. Typically the concentrations in "Current Protocols in Cytometry" should work.
Event rate slows over time	Cells have settled in the lines	Cells settle in the lines after 45-60 minutes of running, resulting in a drop in cell event rate. Stop and save the acquisition. Return the remaining sample, restore the sample volume to 30ul and re-load the sample to continue acquisition. Data can then be appended together in IDEAS®.
	There is a clog or air bubble in the system	Run the purge bubbles script from the instrument drop-down menu. *For more information, see Unstable fluidics (Air or clog in system).*
	Sample syringe is empty	Load a fresh sample.
	Sheath syringe is empty	Load sheath, then go to the instrument drop down and run prime.

Event rate is slower than expected	Sample concentration is low	Make sure the sample concentration is between 10^7 and 10^8 cells/mL. Lower concentrations can be used but this will decrease the cells/second.
	Core is off center	Cropped images will be eliminated from data acquisition and if enough of the images are cropped the event rate can appear lower than normal. Normally this is due to air in the system. Run the purge bubbles script from the instrument drop-down menu. *See solutions for Unstable fluidics* .
	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40 mW. Set the laser powers to maximum and decrease them to prevent pixel saturation.
	Cells are not displayed due to over clipping.	For large diameter cells go to the advanced drop down, select acquisition and check the box labeled keep clipped objects.
Unstable fluidics (Air or clog in system)	Air bubbles in the sample	Make sure a sufficient sample volume is used. To clear the air bubble: Run the purge bubbles script.
		Detergents and foaming agents (such as FBS) can cause bubbles to form in the lines. If these buffers are causing air in the system remove them from the sample and resuspend in dPBS. Run the purge bubbles script.
	Air bubbles in fluid lines	Run the sterilize script, followed by the startup script. Load calibration beads and verify the system runs normally.
	Clog in fluid lines	Filter the sample with a 70 µm nylon cell strainer. Run the sterilize script, followed by the startup script. Load calibration beads and verify the system runs normally.
	Sample is too concentrated	Clumpy and viscous samples cause cavitation in the fluidic lines and create bubbles. Dilute the sample to 1x10^7 cells/mL and strain the cells through a 70 µm nylon mesh. Run the purge bubbles script.
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Compensation wizard fails to complete	The region to collect was set incorrectly	In the wizard verify that 1,000 of "All" cells (or of a region drawn on the appropriate population) are being collected.
	Too many objects are being collected	Set the events to acquire less than 1,000.
	Cells are not fluorescent Cells are stained with more than one fluorochrome	Make sure that the compensation control sample has more than 10% positive events, and are as bright as possible. IgG capture beads or a cell line stained with a single fluorochrome may be used for comp controls. Compensation controls must be a sample with a single fluorochrome label in a single tube. Each fluorochrome must be run separately.
No Images	Camera is not running	Click Run/Setup.
	If the camera is already running	Click Stop to stop the camera, and then click Run-/Setup.
	Imaging is paused	Click Resume.
	Displayed region is incorrect	In the cell view area, select the all population.
	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40 mW. Set the laser powers to maximum and decrease them to prevent pixel saturation.
		Make sure the brightfield lamp is turned on and click Set Intensity.
	Core stream is outside the objective's field of view	Manually find the core stream. In the focus and centering section, move core track left or right to find the core.
	Computer resources are being over used	Close all third party software.
Objects are not centered in the channel	Lateral deviation of the core stream due to air or clog in the system	Run the purge bubbles script from the instrument drop-down menu. *For more information, see Unstable fluidics (Air or clog in system).*
	Autofocus and centering is not tracking properly	In the Focus and Centering section, adjust focus and centering left or right, until the images are centered and in optimal focus.
Plots fail to update, or update slowly	Computer resources are being over used	Close all third party software.
	Too many plots in the template	For optimal plot update rates limit the number of plots to 15.
	Parent population has no qualifying events	Right click on the plot, select graph properties, and change the selected population to "all" or a population that has qualifying events.
	Plots are scaled incorrectly	In the plot tool bar, press the – magnifying glass and rescale the plot.

Data file fails to collect	No events qualify for the region	Make sure there are events going into the collection region by viewing that region in the image gallery and updating the acquisition collection population appropriately.
		Verify the cell concentration is appropriate. $1x10^7$ cells/mL is ideal.
	Computer hard drive is full	Verify the computer hard drive has sufficient room to save the data file. To do this go to Start / Computer / right click on properties and a pie chart showing how much disk space is available is displayed. Backup and delete data to free up disk space.
	Data file collected rapidly	Some samples have high concentrations and acquire faster than the display rate. Check the destination folder and see if the raw data was collected.
	File directory was lost	Collecting data over a downed network or changing the name of the destination folder will cause the instrument to lose the data directory. Verify the data destination folder is accessible using the browse button in the Acquisition Settings section.
	No .rif or .fcs file was created	Go to the file drop down menu and check Generate .rif and or .fcs file.
INSPIRE appears to freeze	Camera is not running	Click Run/Setup.
	If the camera is already running	Click Stop then Run/Setup
	Imaging is paused	Click Resume.
	No objects in the current image view mode	In the cell view area, select the all population.
	A script is running	Wait until the script completes, or if necessary, click Abort Script to prematurely stop the operation.
	The INSPIRE application has crashed	Open the Windows Task Manager by pressing <Ctrl + Alt + Del>. Click the Applications tab. If INSPIRE is 'Not Responding', select the INSPIRE task and click End Now. Restart the INSPIRE application by double clicking the icon on the desktop. If the program restarts, make sure the lasers and brightfield lamp are turned on and then re-establish the core stream. If the application does not start, use the Windows Task Manager to end the INSPIRE task again. Shut the instrument and computer down from the Start menu. Then turn on the instrument as described. If a crash occurs during the day, a complete shutdown is recommended at the end of the day, before running sterilize.